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Characteristics of the Conditioned Medium Produced by CA-12 Lymph Node Stromal Cells

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Abstract CS-21 lymphoma cells that preferentially metastasize to lymph nodes after s.c. inoculation into BALB/c mice were grown *in vitro* in the presence of CA-12 stromal cells isolated from lymph nodes. In order to obtain fundamental data on the identification and characterization of the soluble factors produced by CA-12 stromal cells, the conditioned medium of CA-12 stromal cells that inhibited apoptosis of CS-21 cells was examined. Various analytical treatments revealed that the soluble factors in CA-12 conditioned medium are very sensitive to heat treatment and trypsinization. Moreover CA-12 conditioned medium has an affinity with heparin but not with Con-A. In addition to these, the activity of CA-12 conditioned medium was blocked by H-7, a PKC inhibitor, but the conditioned medium could not induce the differentiation of thymocytes. We concluded that CA-12 conditioned medium contains stromal cell-derived apoptosis-inhibitory molecules that play an important role in proliferation of CS-21 cells by suppressing cell apoptosis.

Key words: Lymph node stromal cells, cell-promoting activity, apoptosis, T-lymphoma

Many papers on the proliferation and differentiation of thymic lymphocytes have been published [15, 19, 20], but little consideration has been given to the possibility that lymph node stromal cells control the proliferation of lymphocytes.

We have previously prepared and characterized mouse malignant T-lymphoma CS-21 (CMS) cells that are highly metastatic to axillary lymph nodes when inoculated s.c. into the right flank of BALB/c mice [17].

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CS-21 cells grew *in vitro* when cells were cocultured with CA-12 lymph node stromal cells but eventually underwent apoptosis after separation from the stromal cells [8]. CA-12 stromal cells exerted bi-directional regulations on the growth of CS-21 lymphoma cells. They promoted the growth of CS-21 cells by direct contact and/or by a soluble factor(s). We recently developrd mAbs that inhibited CS-21 cell adhesion to CA-12 stromal cells. We also found that mAbs MCS-5 and -19, which recognize a Mr 168,000 and a Mr 23,000 protein, respectively, suppressed apoptotic cell death of CS-21 cells even after the cells were separated from CA-12 cells [4]. Therefore, we proposed that cell adhesion molecules, such as CD45 [6] and Thy-1 (CD 90) [5], play an important role in CS-21 cell survival. Besides these adhesion molecules, the effect of soluble factor (s) on apoptosis of these cells has not been investigated until now.

This study covers the acquisition of the preliminary data for the identification and characterization of the conditioned medium of CA-12 stromal cells (CA-12CM) that induces proliferation of CS-21 lymphoma cells. Additionally, we examined whether CA-12CM suppresses the apoptosis of CS-21 cells.

MATERIALS AND METHODS

Cell Lines and Reagents

CS-21 lymphoma cells and CA-12 stromal cells were cloned from Lymph A cells. Lymph A cells were originally established from a spontaneously developed tumor mass in the auxillary lymph node of BALB/c mice. Although CA-12 stromal cells could not form a tumor mass following s.c. inoculation in BALB/c mice, CS-21 cells formed a tumor mass and selectively metastasized to the lymph node. CA-12 stromal cells have properties similar to those of stromal cells in lymph nodes. CS-21

cells, however, express Thy-1 and CD4 antigen but not CD8, like mature helper T-lymphocytes (data not shown). Some other fibroblasts were also used as follows: MLF, mouse lung fibroblasts; MKF, mouse kidney fibroblasts; MoCoF, mouse colon fibroblasts; ST2, bone marrow stromal cells [13]. H-7, and HA-1004 were purchased from Seikagaku Co. (Tokyo, Japan). All other reagents are commercially available.

Separation of CS-21 Cell-enriched Population

CA-12 stromal cells (1×10^5 cells) were plated in a culture dish (10-cm diameter) containing 10 ml of RPMI medium supplemented with 5% heat-inactivated fetal bovine serum (Biocell, Carson, CA, U.S.A.) and kanamycin at 100 μg/ml and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After 16 h of incubation, CS-21 lymphoma cells (5×10^5 cells/ml) were added to the CA-12 cell culture and allowed to grow further. After 5 or 6 days, CS-21 cells were harvested with CA-12 stromal cells from the dishes by pipetting. The mixed cell suspension was first centrifuged at $50\times g$ for 5 min to precipitate large CA-12 stromal cells. The supernatant was transferred to a new 50-ml tube and centrifuged further at $800\times g$ for 5 min to collect small CS-21 cells. The precipitated cells were resuspended in RPMI growth medium and incubated for 1 h in a culture dish to allow attachment of the remaining adhesive CA-12 stromal cells to the dishes. The unattached cells were carefully transferred to a new 50-ml tube and centrifuged at $50\times g$ for 5 min. The supernatant was harvested and centrifuged again at $800\times g$ for 5 min in order to collect CS-21 cells [8].

Preparation of the Conditioned Medium of CA-12 Stromal Cells

CA-12 stromal cells were seeded at a concentration of 5×10^4 cells/ml and then cultured for 48 h until reaching to the subconfluent condition. The medium was changed with pre-warmed PRMI growth medium containing 5% FBS. After a 24-h incubation, the conditioned medium (CA-12CM) was harvested and immediately used for the further study.

Measurement of DNA Synthesis.

CS-21 cells (5×10^4 cells/ml), harvested as described above, were incubated in 1 ml of the RPMI growth medium containing various concentrations of the conditioned medium at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Fifty-four hours later, the cells were pulse-labeled with 1 μCi of [^{3}H] thymidine for 18 h and harvested. The radioactivity incorporated into cellular DNA was measured using an LKB liquid scintillation counter (Beckman, Irvine, CA, U.S.A.).

Preparation of Thymocytes

BALB/c mice (3- to 4-week-old) were killed for thymus preparation. From the thymus, the cell clumps were centrifuged at 800 rpm for 5 min and then added to 20 ml of 0.83% NH_4Cl/20 mM HEPES (pH 7.2) on ice for 5 min. To remove NH4Cl, an additional 20 ml of RPMI medium was added and the mixture was centrifuged again. Finally, the thymocytes were suspended in RPMI medium and counted. Viable cells were used for the differentiation of $CD4^+CD8^+$ thymocytes into a $CD4^-CD8^+$ or $CD4^+CD8^-$ thymocytes [16].

FACS Analysis

In order to examine the capability of CA-12 stromal cells to induce differentiation of thymocytes, thymocytes were cultured with CA-12 stromal cells for 1 day and analyzed using a FACScan (Beckton Dickinson). In brief, the intermediate double positive thymocytes (1×10^6 cells/ml) were cultured on a monolayer of CA-12 stromal cells or in the conditioned medium of CA-12 stromal cells in 24 well plates for 24 h. After harvesting and washing twice with PBS, anti-CD4 mAb was added and incubated for 1 h. After washing twice, anti-rat IgG-FITC (Cappel, Cochranville, PA, U.S.A.) was added to the wells at a 500-fold dilution to RPMI medium for 30 min on ice. PE labeled anti-CD8 mAb (Pharmingen) was also added and incubated as was done with FITC [12]. Finally, the stained thymocytes were analyzed with LYSIS II software program (Beckton Dickinson).

Analysis of DNA Fragmentation

CS-21 cells (1×10^6 cell/ml) were suspended in 20 μl of 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.5 mg/ml proteinase K (Sigma St. Louis, MO, U.S.A.). After incubation at 50°C for 1 h, a 10-μl aliquot of 0.5 mg/ml RNase A solution was added to a mixture of 10 μl of the 70℃-preheated solution containing 10 mM EDTA (pH 8.0), 1% (w/v) low-melting point agarose (Sigma), 0.25% bromophenol blue, and 40% sucrose. The DNA was analyzed by electrophoresis in 2% agarose gels followed by ethidium bromide staining and photographing on a UV illuminator.

RESULTS

Effect of Conditioned Medium of Various Tissue-derived Fibroblasts on CS-21 Cell Growth

The effect of conditioned medium from various tissue-derived fibroblasts on CS-21 cell growth was investigated. Various stromal cells were seeded at 1×10^4 cells/ml. The following day CS-21 cells were added at a concentration of 5×10^4 cells/ml and then incubated. The viable CS-21 cells were counted everyday over a four

day period. Among other tissue-derived stromal cells, CA-12 lymph node stromal cells (CA-12CM) exhibited the greatest effect on CS-21 cell growth (Fig. 1A). In contrast, the addition of conditioned medium from cultures of various stromal cells produced fewer viable cells when compared with coculture conditions (Fig. 1B). Next, the thymidine incorporation of CS-21 cells in the presence of various conditioned medium was examined. Figure 1C shows that CA-12CM is most effective for CS-21 cell proliferation. These results suggest that CA-12CM has an activity to induce CS-21 cell growth.

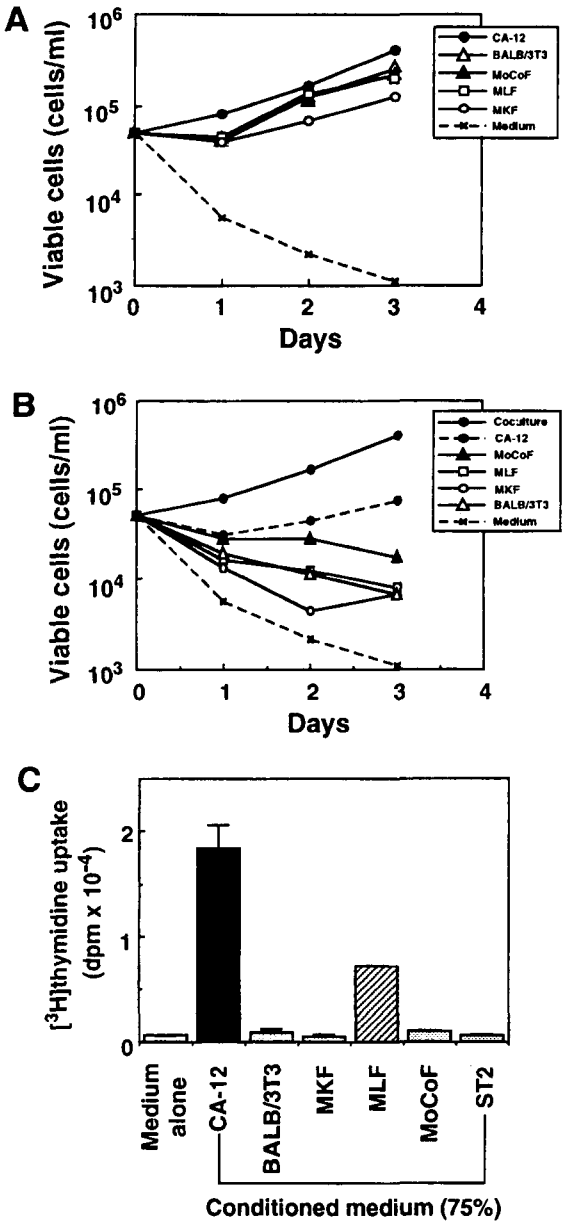

Fig. 1. Stimulation of CS-21 cell growth.

(A) Effects on CS-21 cell growth by cell contact with various mouse fibroblasts. CS-21 cells were separated from CA-12 stromal cells, as described in Materials and Methods. CS-21 cells were cultured with various fibroblasts (1×10^4 cells/ml growth medium) which had been plated before 24 h. (B) Effects on cell growth by conditioned medium (75%). The number of viable cells was determined by trypan blue exclusion method. (C) Effect of conditioned medium of some fibroblasts on CS-21 cell growth. Each conditioned medium was prepared as follows; fibroblast cells were seeded at a concentration of 5×10^4 cells/ml. When the cells were grown in 60~70% cell confluence, RPMI medium was added and reincubated for 24 h. The conditioned medium was harvested and tested for its effect on CS-21 cell proliferation by [^{3}H]thymidine incorporation assay.

Comparison of Culture Conditions and the Stability of CA-12CM

In order to characterize and purify the soluble factors produced by CA-12 cells, the effects of stabilizing agents, cell density, and culture time on production of CA-12CM were investigated. Ethylene glycol is a stabilizing agent for enzyme purification. Bovine serum albumin (BSA) is also used as a stabilizer of proteins such as growth factors. The addition of BSA in CA-12CM lengthens the activity of CS-21 cell

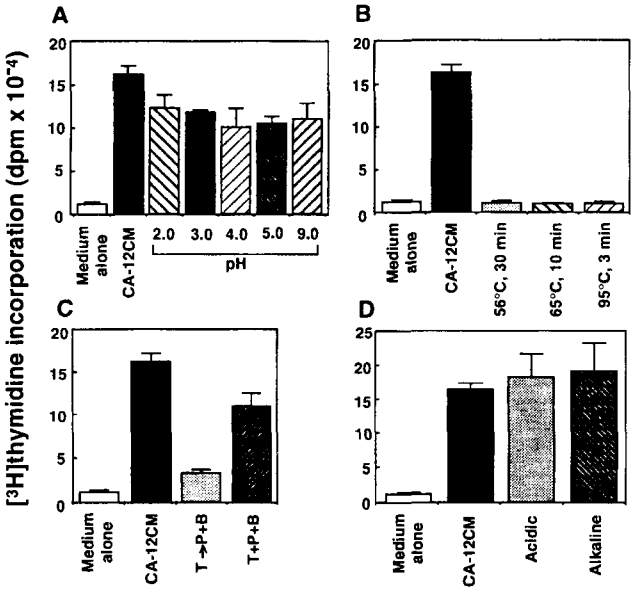

Fig. 2. Comparison of stability of CA-12CM activity by various pH (A), temperature (B), trypsin (C), or acidic and alkaline (D) treatments.

(A) Centricon C-100 concentrated CA-12CM was added to threefold volumes of either 0.2 M glycine-HCl for pH 2.0, 0.2 M sodium acetate for pH 3.0~5.0 or 0.2 M Tris-HCl for pH 9.0, and incubated at 20°C for 5 h. (B) The concentrated CA-12CM was heated as indicated and then dialyzed. (C) The concentrated CA-12CM was either treated with trypsin [T, at a concentration of 15 μg/ml] at 37°C for 2 h followed by the addition of PMSF [P] at a final concentration of 1 mM plus BSA [B], added simultaneously with the same amount of trypsin, PMSF plus BSA or treated with dithiothreitol (DTT, at a final concentration of 10 mM) at 25°C for 2 h. After treatment, samples were dialyzed against serum-free RPMI. (D) Concentrated CA-12CM was added to ninefold volumes of 1 M HCl or 1 M NaOH. After incubation for 30 min at 4°C, the sample was neutralized, dialyzed, and then assayed.

growth, whereas ethylene glycol has cytotoxicity against CS-21 cells (data not shown). In addition, when CA-12 stromal cells were seeded at a concentration of 1×10^4 cells/ml, the relative growth of CS-21 cells was most optimal (data not shown). Because the stability of CA-12CM is a crucial determinant for identifying the CS-21 cell-promoting soluble factor, the stability of CA-12CM which was harvested by various treatments was examined (Fig. 2A~D). It was revealed that pH treatment (Fig. 2A), alkaline and acidic treatment (Fig. 2D) did not affect the promoting activity of CS-21 cells, but the treatment of heat or trypsin radically reduced the growth promoting activity of CS-21 cells.

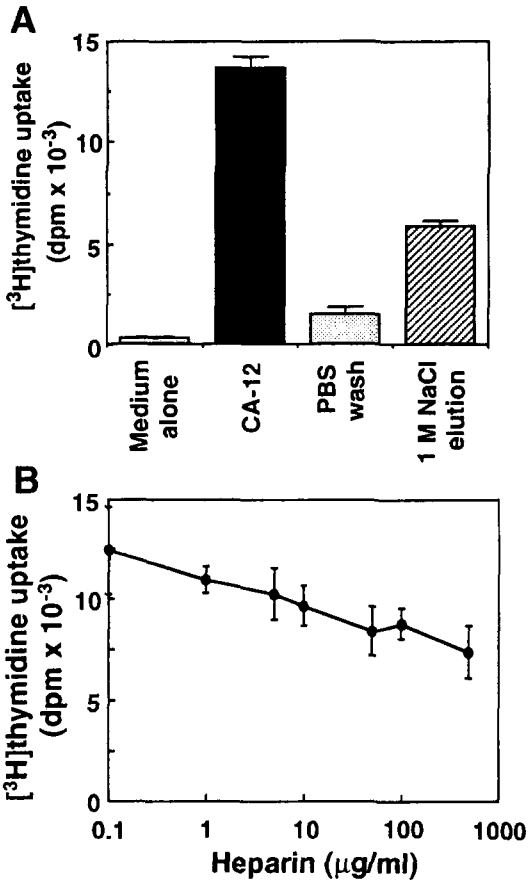

Fig. 3. CA-12CM has an affinity with heparin.
(A) CA-12CM collected from subconfluent plates of CA-12 stromal cells, as described in Materials and Methods, was applied to a heparin-sepharose column packed with CL-6 (Pharmacia Biotech, Uppsala, Sweden). After washing with serum-free medium three times, the column was eluted with PBS or with PBS containing 1 M NaCl. CS-21 cell proliferation was assayed by [^{3}H]thymidine incorporation. (B) Effects of heparin on CS-21 cell growth induced by CA-12CM (75%). CS-21 cells (1×10^5 cells/ml) were cultured in the presence of various concentrations of heparin. After incubation for 48 h, CS-21 cells were labelled with 1 μCi of [^{3}H]thymidine for the last hour and harvested, as described in Materials and Methods. Bars indicate SD values of triplicate determinations.

Effects of Heparin on CS-21 Cell Growth

To identify the soluble factors which affect CS-21 cell growth, the properties of the soluble factors were examined. Among the molecular media for chromatographic analysis, we examined whether CA-12CM has an affinity with heparin (Fig. 3A) [9]. In Fig. 3A, the CS-21 cell proliferating activity can be eluted to 1 M NaCl (45% of total activity). As shown in Fig. 3B, CA-12CM had a weak affinity for heparin (37.5% reduced). On the other hand, it is known that Con A binds to some glycosylated proteins [1]. Heparin was added to the wells and incubated for 48 h with CS-21 cells. It was found that the soluble factors in CA-12CM did not bind to Con A (data not shown). These results suggest that CA-12CM has an affinity for heparin but not for Con A.

Activation of PKC by CA-12CM

By using metabolic inhibitors [3, 18], we can determine how inhibitors block a signal transduction pathway induced by a growth factor. The signal transduction pathway of T-lymphoma cells remains to be studied. It is well known that activation by PMA, a PKC-activating compound, is blocked by PKC inhibitors. In order to investigate the mechanism of CS-21 cell growth induced by CA-12CM, we used H-7, a PKC inhibitor, and compared it to a control (HA-1004), using and with PMA as a positive control. As shown in Figure 4, it was found that the soluble factor (s) produced from CA-12 stromal cells was blocked by H-7. This result means that CA-12CM may contain a soluble factor (s) that activates PKC in CS-21 cells.

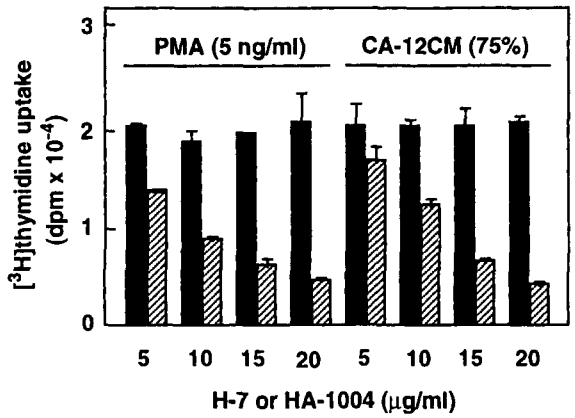

Fig. 4. Inhibition of protein kinase C activity by H-7, a PKC inhibitor.
CS-21 cells were added to 24 well plates to a concentration of 1×10^5 cells/ml with various concentrations of H-7 as a PKC inhibitor (shaded columns), and HA-1004 as a negative control (black columns). The concentration of PMA was 5 ng/ml. CA-12 conditioned medium (450 μl) was added. Total incubation volume was 600 μl with 150 μl cells at a concentration of 4×10^5 cells/ml.

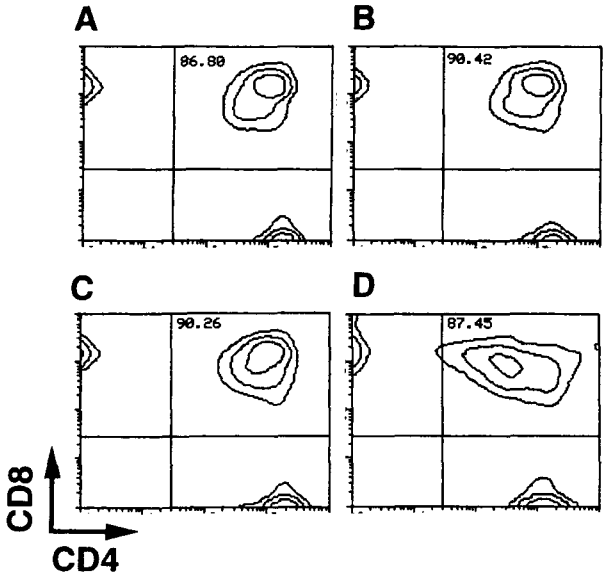

Fig. 5. Differentiation of $CD4^+CD8^+$ thymocytes into a $CD4^-CD8^+$ or $CD4^+CD8^-$ thymocytes.
The thymus of BALB/c mouse was used as a source of intermediate thymocytes. Generation of $CD4^-CD8^+$ or $CD4^+CD8^-$ intermediate thymocytes was analyzed using a FACScan. Double positive thymic cells were cultured alone (A), on monolayers of CA-12 cells (B), on a Transwell (C) in 24 well culture plates for 24 h. Thymocytes recovered 1 day after culturing were stained and analyzed for two-color fluorescence. The numbers on the figures are the percentages of cells stained by each of FITC and PE. See Materials and Methods in detail.

Failure to Induction into Single Positive Thymocytes by CA-12CM

We next examined whether the differentiation of double positive thymocytes ($CD4^+CD8^+$) through single positive thymocytes into more stages can be induced by CA-12CM. Thymocytes from 3- to 4-week-old BALB/c mice were collected and analyzed by FACS. The major population of fresh thymocytes was determined as $CD4^+CD8^+$ although a minor single positive population was observed. Figure 5 shows that culture of double positive thymocytes on CA-12 stromal cells (Fig. 5B) resulted in no generation of a number of single positive cells. These results suggest that the soluble factor (s) in CA-12CM is not concerned with differentiation into single positive populations.

Apoptosis Suppression by CA-12CM

There are several reports that growth factors inhibit apoptosis of some haematopoietic cells. So we examined whether the apoptosis inhibition of CS-21 cells can occur by the addition of CA-12CM. Figure 6 shows that apoptosis inhibition was dependent on the concentration of CA-12CM (lane 2~4). Below 37.5% addition of CA-12CM failed to inhibit apoptosis of CS-21 cells. Moreover, when CA-12CM was concentrated with Centricon concentrators (cut-off of molecular weight

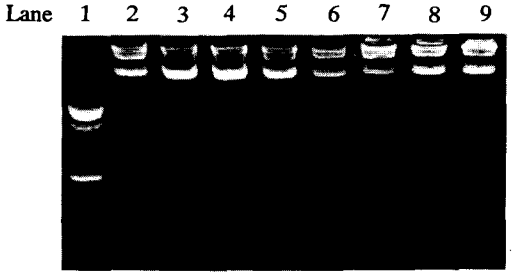

Fig. 6. Partial inhibition of CS-21 cell apoptosis by conditioned medium from CA-12 lymph node stromal cells.
CS-21 cells were isolated from the cultures with CA-12 stromal cells and then 1×10^6 purified CS-21 cells were incubated with fresh RPMI growth medium (lane 5), anti-Thy-1 mAb (10 μg/ml of MCS-19) (lane 6), or 75% (lane 2), 37.5% (lane 3), and 7.5% (lane 4) of CA-12 conditioned medium, and 75% (lane 7), 37.5% (lane 8), and 7.5% (lane 9) of CA-12 conditioned medium concentrated with Centricon C-100 (Amicon). The DNA was isolated from each sample and analyzed for DNA fragmentation by electrophoresis in 2% agarose gel plates. Lane 1 indicates the 100-bp DNA size marker.

10,000), the activity remained in the pass-through fraction of molecular mass cut-off of 10 kDa. Interestingly, this fraction had an antiapoptotic activity (lane 7~9). These results indicate that CA-12CM contained antiapoptotic soluble factors.

DISCUSSION

Proliferation and differentiation of hematopoietic stem cells depend on the presence of positive and negative signals [10]. These signals are mediated by growth factors and by cell contact [10].

Despite the abundant documentation for the role of growth factors and the adhesion molecules in metastatic process, no report has been made about the mechanisms by which growth factor (s) and/or adhesion molecule (s) excreted from stromal cells influence metastatic T-lymphoma cells in the lymph node. Because growth and apoptotic cell death of T-lymphoma CS-21 cells are largely affected by cytokines and adhesion molecules, the identification of the factors provides valuable informations about CS-21 supported cell growth by CA-12 stromal cells.

Apoptosis is a controlled process by which an individual cell within an organism is eliminated. In multicellular organisms, homeostasis is achieved through a continual balance between cell proliferation, quiescence, differentiation and death. Recently, apoptosis is a lively research field, especially in tumor biology. While loss of proliferative control is a common feature of cancers, an unregulated propensity for apoptosis may lead to degenerative diseases. Some tumor cells lose the ability to die by apoptosis. In solid tumors, cells that escape growth

control and lose responsiveness to signals that would trigger apoptosis contribute to the net accumulation of solid tumor mass.

On the other hand, it was proposed that CA-12 stromal cells exert bi-directional regulations on the growth of CS-21 lymphoma cells [4~6]. They promote the growth of CS-21 cells by direct cell-cell contact or by a soluble factor (s). In the course of investigating the role of cell adhesion molecules, some mAbs raised against CS-21 cell surface molecules that partially inhibited adhesion of lymphoma cells to lymph node stromal cells were developed [4].

We demonstrated that CS-21 cells proliferate in the presence of conditioned medium produced from CA-12 stromal cells. In this study, to elucidate the interaction between CA-12 lymph node stromal cells and CS-21 lymphoma cells that enhance the growth of the lymphoma cells, we first examined whether the soluble factors of CA-12 stromal cells mediate the control of CS-21 cell growth which preferentially metastasized. The conditioned medium from various fibroblasts indicated that CA-12CM is the most effective on CS-21 cell growth. Because CS-21 cells were killed by apoptosis without CA-12CM, some molecules of CA-12CM play an important role in survival of CS-21 cells.

When CS-21 lymphoma cells were cultured in the presence of mouse IL-1, mouse IL-2, mouse IL-3, mouse IL-4, mouse IL-5, mouse IL-6, mouse IL-9, mouse IL-10, bFGF, mouse SCF, and/or human NGF, these cytokines and growth factors, however, could not stimulate the growth of CS-21 lymphoma cells (data not shown). Other cytokines or growth factors might be involved in the growth promotion. Recently, it was reported that the addition of cysteine to the culture medium greatly enhanced the survival of rat chondrocytes [7]. Moreover, when the conditioned medium from rat chondrocytes cultures was fractionated by ultrafiltration using a membrane with the molecular mass cut-off at 10 kDa, the survival-promoting activity was recovered in the concentrate and not in the filtrate. But our cell system showed that the CS-21 cell promoting activity was contained in the concentrated fraction of the molecular mass cut-off of 100 kDa and in the pass-through fraction of the molecular mass cut-off of 10 kDa (data not shown). These results suggest that CA-12CM has, at least, two factors, not one, to support CS-21 cell growth.

There are increasing numbers of examples where several extracellular signaling molecules have been shown to collaborate to promote cell growth in culture [2]. Not one, we suggest, but several soluble factors from CA-12CM enable CS-21 cells to proliferate and to avoid apoptosis, which may help CS-21 cells to metastasize in lymph nodes *in vivo*.

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